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Contents

ORIGINAL ARTICLES

ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

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ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

Under the above caption will be published from time to time comments criticisms and suggestions on technical procedures; minor contributions such as laboratory aids and short cuts which are not considered sufficiently important to warrant a formal paper; and queries.

Obviously comments and criticisms should be signed; queries should be signed but names will be withheld on request; full credit will be given those who contribute laboratory aids, short cuts and the like.

An attempt will be made to obtain answers from authoritative sources to the queries submitted. It must be emphasized that the views expressed in this department are not the opinions of any official body.

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TECHNIQUE OF BLOOD GROUPING TESTS PRELIMINARY TO BLOOD TRANSFUSIONS*

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Blood grouping is usually regarded as such a simple procedure that a special article describing the technique may seem superfluous. As a matter of fact, there are a number of pitfalls in the test not always recognized and guarded against, so that errors in grouping are still too frequently made. Accordingly, in this article methods of avoiding the common sources of error will be described. The technique given is so simple that workers with barely more than a week's experience can carry out the tests accurately.

PRINCIPLE OF BLOOD GROUPING

The existence of the human blood groups, first described by Landsteiner in 1900–01, depends on the presence in the red blood cells of two agglutinogens, A and B, and in the serum of two corresponding isoagglutinins, α (or anti-A) and β (or anti-B). The group of any individual is hence determined by the agglutinogens and agglutinins present in the blood (see table 1).

It is somewhat unfortunate that two systems of nomenclature, those of Moss and Jansky, were introduced, because the transposition of Groups I and IV, as shown in table 1, has often led to confusion and transfusion accidents. It is therefore of essential importance that the blood grouping report *state the classification used* (Moss or Jansky).

Since the international nomenclature is based on the agglutinogen content of the red blood cells, it is more readily comprehended and remembered; and since it is the classification in general use in scientific publications, it will be used in this paper.

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The blood cells of the individual whose group is to be determined must be tested for the two agglutinogens A and B. Two different reagents are required, one containing the agglutinin α (or anti-A) and the other agglutinin β (or anti-B). Since the serum of group B individuals contains α agglutinin alone, such serum is used as the anti-A reagent; serum of group A individuals, which contains anti-B agglutinin is used for testing for agglutinogen B. To determine the blood group, therefore, all that is necessary is to test the blood cells with two standard sera taken from group A and group B individuals, respectively. If the unknown cells are not agglutinated by either serum, the individual in question belongs to group O; if only by B serum, the group in question is A; if agglutination only occurs with the group A

TABLE 1

CLASSIFICATION AND COMPOSITION OF THE LANDSTEINER BLOOD GROUPS

INTERNATIONAL NOMENCLATURE	JANSKY NUMBERING	MOSS NUMBERING	CELLS (AGGLUTINOGEN)	SERUM (AGGLUTININ)
O	I	IV	—	α and β
A	II	II	A	β
B	III	III	B	α
AB	IV	I	A and B	—

serum, the group is B; if agglutination is produced by both reagents, the group is AB.

Landsteiner pointed out that those isoagglutinins are always present in the serum of an individual for which there are no corresponding agglutinogens in the red blood cells (Landsteiner's rule). Thus, individuals of group O have both isoagglutinins α and β in their sera; individuals belonging to group A have β but not α, etc. Accordingly, it is possible to confirm the results of the grouping tests performed on a person's blood cells by determining the isoagglutinin content of his serum. This is done by testing the unknown serum against standard cell suspensions of group A and B respectively. If both sorts of cells are agglutinated, both agglutinins anti-A and anti-B are present in the serum, and the serum must have been derived from a group O individual; similar reasoning applies for the sera of the other

three groups. *This confirmatory test has no value, however, in the case of very young infants,* since while the agglutinogens are demonstrable at birth, the agglutinins are not fully developed for several months after birth. In such instances the group is determined from the reactions of the cells alone.

After the group of the patient has been determined, a donor of the same group can be obtained by calling a professional donor agency (or in institutions which use stored blood, by applying to the "Blood Bank"). Then all that is required is to carry out the cross-matching tests; namely, the serum of the patient is mixed with the cells of the donor, and the cells of the patient with the serum of the donor. If no agglutination occurs in either mixture the donor is considered compatible. Before the donor is used for the transfusion, however, he must be given a physical examination, a history of past and present illness taken (in particular, any history suggestive of syphilis or malaria must be excluded), and his blood must be tested for its hemoglobin content and submitted to one of the approved serological tests for syphilis.

Summarizing, in the selection of a suitable donor three essential criteria must be fulfilled:

1. He should preferably be of the same group as the patient.[1] In this connection, as has already been emphasized, it is necessary that *the same method of blood typing and the same classification of blood types be used for both donor and recipient.*

2. The compatibility of the donor's cells and recipient's serum, and of the recipient's cells and donor's serum, must be ascertained by cross-matching.

3. The donor must be healthy and free from obvious evidence of communicable disease, particularly syphilis and malaria.

TECHNIQUE OF BLOOD GROUPING

For the performance of blood grouping tests one requires the blood of the individual to be examined, standard testing sera, standard human blood cells of known groups, normal saline solution, citrate solution, glass slides, coverslips, test-tubes, pipettes, nipples and a microscope.

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[1] The use of so-called "universal" donors will not be discussed here. Most authorities favor blood of the same group as the patient in preference to group O blood.

Standard sera

The most important step in the blood grouping tests is to procure specific standard sera of high potency. This, unfortunately, is the step to which not enough attention is ordinarily paid. Two methods in rather common use to collect typing sera are mentioned here only to be condemned. One is to collect blood from Wassermann specimens, group the cells, and pool all A or B sera; the other is to bleed known A and B individuals at random. Since the average potency of sera taken at random is low, it is clear that *these procedures are not advisable.*

It is essential that typing sera be of high potency and therefore *the titer of each A and B serum collected must be determined* and standard testing sera selected from those *showing a high titer of isoagglutinins.*

Method of determining titer of typing sera

In titrating *group A sera* the following procedure is recommended:

1. Five dilutions of each serum are made with normal saline solution, namely, 1:1 (undiluted serum), 1:10, 1:20, 1:40, and 1:80. In making the dilutions, a piece of cotton is inserted into the pipette, to prevent the saliva from entering the pipette and possibly inhibiting the action of the serum.[2] The dilutions also may be made by counting drops with the aid of a capillary pipette and nipple.

2. One drop of each dilution is placed in a corresponding test tube and to each tube one drop of a suspension of B cells is added.

3. After 1 hour the mixtures are examined for agglutination, and the reciprocal of the highest dilution in which distinct agglutination (i.e., visible to the naked eye) occurs is the titer of the serum. *Only those sera giving distinct reactions in dilutions of 1:20 are suitable for use for grouping tests.*

In the case of *group B sera*, the same method is followed but, in addition, cognizance must be taken of the fact that at least 2 different sorts of A cells exist, namely, A_1 and A_2, the latter being less sensitive to agglutination by most sera than the former. If feasible, therefore, such sera should be titrated against A_2 cells as well as A_1 cells, and should be considered suitable for use only if they agglutinate the A_2 cells in dilution of at least 1:20. If the subgroup of the A cell suspension used for the titration is not known, no group B testing serum should be considered satisfactory unless it agglutinates the cells in a dilution of at least 1:40.

Collection of typing sera

After individuals have been found whose sera have a sufficiently high isoagglutinin titer blood can be taken from their veins with a large syringe and

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[2] Saliva of individuals of groups A, B and AB frequently contains group-specific substances which neutralize the corresponding isoagglutinins.

needle under sterile conditions, distributed among a number of sterile tubes and allowed to clot. The clots are allowed to contract of their own accord, or they can be rimmed and the serum separated by centrifuging. The serum is then transferred to sterile 1 cc. ampoules which are sealed and placed in the water bath at 56°C. for 30 minutes in order to inactivate them and at the same time to help in sterilizing the sera in case of accidental contamination. This step is important because fresh potent sera containing complement may hemolyse red cells instead of agglutinating them, and during the process of hemolysis there is inhibition of agglutination,[3] so that the reaction might be misinterpreted as negative. By inactivating the serum this source of error is eliminated. The ampoules are then carefully labelled with the group and date and stored in the refrigerator.

Whenever a fresh ampoule is opened the serum should be retested against standard cells A and B to confirm the specificity and potency of its reactions.

Testing sera can be purchased from commercial houses, but inasmuch as the person doing the actual grouping tests must assume full responsibility, he must satisfy himself of the specificity and potency of such sera before using them. This is emphasized because cases have occurred in which inactive or feebly active sera were distributed or where the sera were incorrectly labelled. The writer prefers not to put up sera in capillary tubes holding enough for only a single test, which makes satisfactory checking of the reagents more difficult.

STANDARD CELLS

For the proper execution of blood grouping tests, it is necessary to have available individuals whose blood groups are known, so that their blood cells can serve as controls and for testing the sera of the individuals to be grouped. For ordinary purposes suspensions of group A and group B cells alone are sufficient. For special purposes, however, it is advantageous to have at hand also group O cells and group A cells of both subgroups, A_1 and A_2.

Suspensions can be prepared by taking the blood from the finger directly into tubes containing normal saline solution, to which may also be added a

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[3] On account of this property of fresh high-titered sera, a recent book on clinical microscopy advises against the use of "too strong" sera for typing. In the writer's experience, the more potent the sera the better are the results, and as is pointed out above, this difficulty is obviated by inactivating the sera.

few drops of 3.8 per cent citrate solution, to guard against the possibility of clotting. Enough blood is taken to form a 1 to 2 per cent suspension, in terms of blood sediment. It is recommended to use fresh blood suspensions; at any rate, not older than 1 to 2 days, since the sensitivity of the cells may diminish rather rapidly when they are kept in saline solution. Moreover, blood cells stored as suspensions at times become "panagglutinable," should the suspension become infected with certain special bacteria having the property to bring about this peculiar change in the red blood cells.

For preserving blood, it is best to keep it as a clot in the refrigerator without the addition of any preservative. Accordingly, if there is difficulty in obtaining standard blood cells, the following method of storing them may prove helpful, and is used by the writer.

1. Two cubic centimeters of blood are obtained by venepuncture or from a deep puncture of the finger, and distributed among 10 small tubes.

2. The blood is allowed to clot, the tubes corked, labelled and dated, and stored in the ice-box. Blood kept in this way is usually good for 7 to 10 days.

3. If standard cells are needed on any particular day, a small amount of saline solution is added to a tube, the blood clot shaken up in the saline and the mixture allowed to stand for a few moments so that coarse particles settle out. The supernatant smooth suspension is adjusted to the proper concentration (1 to 2 per cent) and used for the tests.

Citrate and saline solutions

The citrate solution used for the grouping tests is prepared very simply by dissolving 3.8 grams of C.P. sodium citrate in 100 cc. of distilled water. The saline solution is made by dissolving 9 grams sodium chloride in 1000 cc. of distilled water. It will be found convenient in the case of the saline solution to prepare a stock 10 per cent solution, from which enough normal saline solution for the day's need can be prepared by diluting 9-cc. portions up to 100 cc. with water.

BLOOD OF THE INDIVIDUAL BEING GROUPED

If there is no urgency in ascertaining the group of an individual, as when grouping a professional donor or patients who are not acutely ill, the blood may be taken as for a Wassermann test. After it has clotted, the clot is rimmed and the serum separated by centrifuging. A blood suspension is then prepared by shaking the clot in normal saline solution, the strength of the suspension being adjusted to approximately 1 to 2 per cent in terms of blood sediment.[4] When it is not intended to make serum tests blood

[4] The concentration is specified in terms of blood sediment instead of whole blood to allow for variations in the red cell count, as in anemic cases. The strength of the blood suspension is judged by the eye.

suspensions may be prepared directly by suspending a few drops of blood in normal saline solution.

The following rapid method of obtaining cell suspensions and plasma suitable for pre-transfusion blood grouping tests has the advantage that only a small amount of blood is needed.

1. Into a small tube 1 or 2 drops of citrate solution are placed and to this are added nine times as many drops of blood taken from the finger. In the case of infants, a free flow of blood can be obtained by first immersing the foot in warm water, applying a tourniquet (not too tightly), and then puncturing the heel.

2. The citrated blood is centrifuged and the supernatant plasma pipetted off into a second tube. To the sediment enough saline is added to make a 1 to 2 per cent blood suspension.

Some workers employ whole blood taken directly from the finger or whole defibrinated blood instead of thin suspensions. This procedure has led to errors, because of rouleaux-formation, and the lower sensitivity of the tests made in this manner.

THE GROUPING TESTS

A number of different techniques for grouping blood has been suggested, but *the actual method selected is of less consequence than the safeguards with regard to the potency and specificity of the typing sera.* Landsteiner recommends that one drop each of cell suspension, serum and saline be mixed in small test-tubes (inside diameter 7 mm.) and the mixtures allowed to stand at room temperature. With this method agglutination is usually visible within a few minutes, but the reactions may not reach their maximum for about an hour. This method is ideal when a large number of blood specimens have to be tested at one time. Where speed is of importance, the reactions can be accelerated, as Schiff has recommended, by centrifuging the tubes in order to pack the cells. Then the tubes are gently shaken, and if the reaction is positive clumps will persist and float about in the clear supernatant fluid, whereas in negative reactions the cells readily separate to form an even suspension.

In most institutions the grouping tests preliminary to transfusion are carried out on glass slides. Two slides are needed, one for testing the patient's cells with known sera, and a second for

the confirmatory tests on the patient's serum against known cells (figure 1). The confirmatory tests are recommended because if

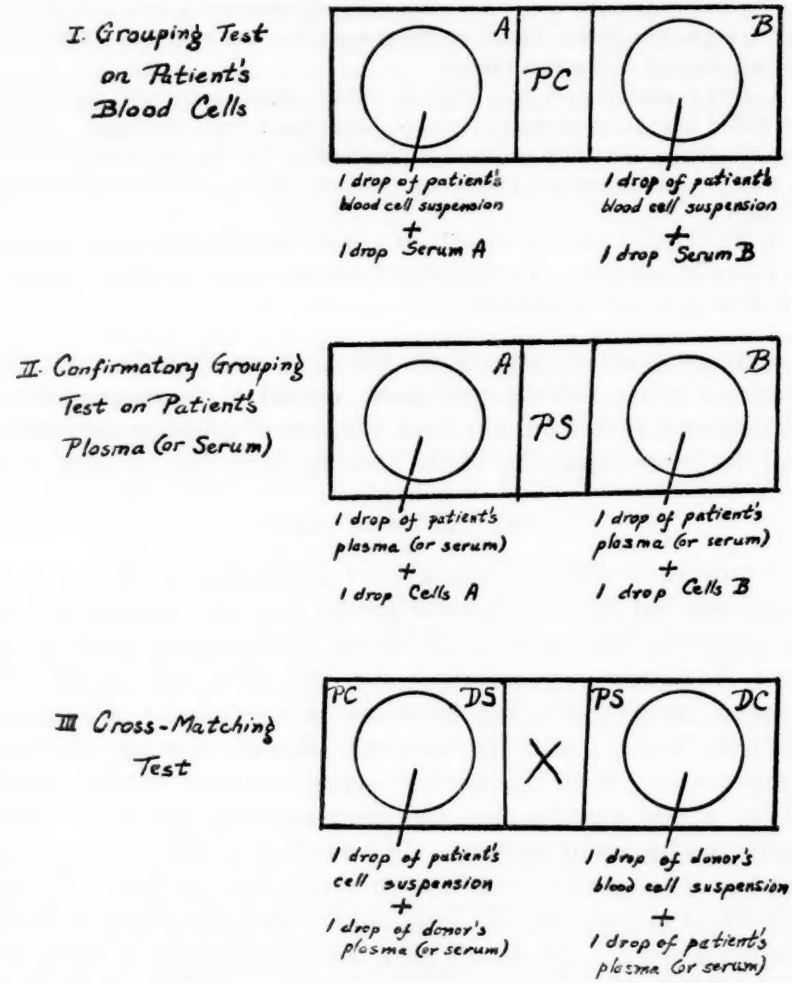

FIG. 1. DIAGRAM ILLUSTRATING TECHNIQUE OF GROUPING AND CROSS-MATCHING BLOODS OF PATIENT AND DONOR, BEFORE BLOOD TRANSFUSIONS

these are always carried out the chance of making mistakes will be reduced to a minimum. However, a survey of American

hospitals reveals that, with few exceptions, only the cells and not the serum are tested, even in New York City, despite the fact that the New York City Sanitary Code for 1930 requires that both tests be done.

The actual tests are very simple to perform.

A. On one slide the grouping tests of the patient's cells are carried out:

1. One drop of the cell suspension is placed on each end of the glass slide, and on the left side is added 1 drop of group A serum (diluted 1:2 with saline), and on the right, 1 drop of group B typing serum (diluted 1:2).[5]

2. Each mixture is then stirred, spread out over an area approximately 2 centimeters in diameter, and the slide is then tilted back and forth for 2 to 3 minutes.

3. A coverslip is dropped over each mixture and the reactions read with the naked eye and under the low power of the microscope. The interpretation of the reactions has already been outlined above, and the actual macroscopic appearance of the agglutination reactions for each of the four blood groups is shown in figure 2.

B. On a second slide the patient's plasma (or serum) is tested against the standard blood cell suspensions.

1. On each end of the glass slide is placed 1 drop of the patient's plasma (or serum); to the left hand drop is added 1 drop of A cell suspension and to the right drop, 1 drop of group B cell suspension.

2. These mixtures are treated as described above except that more time is allowed for the reactions to take place and the reactions are read under the low power of the microscope.

The results of the serum tests should confirm the results of the tests carried out on the cell suspensions except in the case of infants, when, as has already been pointed out, the agglutinins may not be fully developed. Only in rare cases (in the order of about one out of two to three thousand individuals) are agglutinins lacking in older children and adults. Therefore, failure of the two tests to check one another in individuals more than 2 years of age can usually be considered an indication that some mistake in technique has been made.

[5] The grouping sera are diluted before use to prevent pseudoagglutination, which is more apt to occur with the slide technique than in tubes. This slight dilution will usually eliminate that source of error and should not appreciably diminish the strength of the reactions.

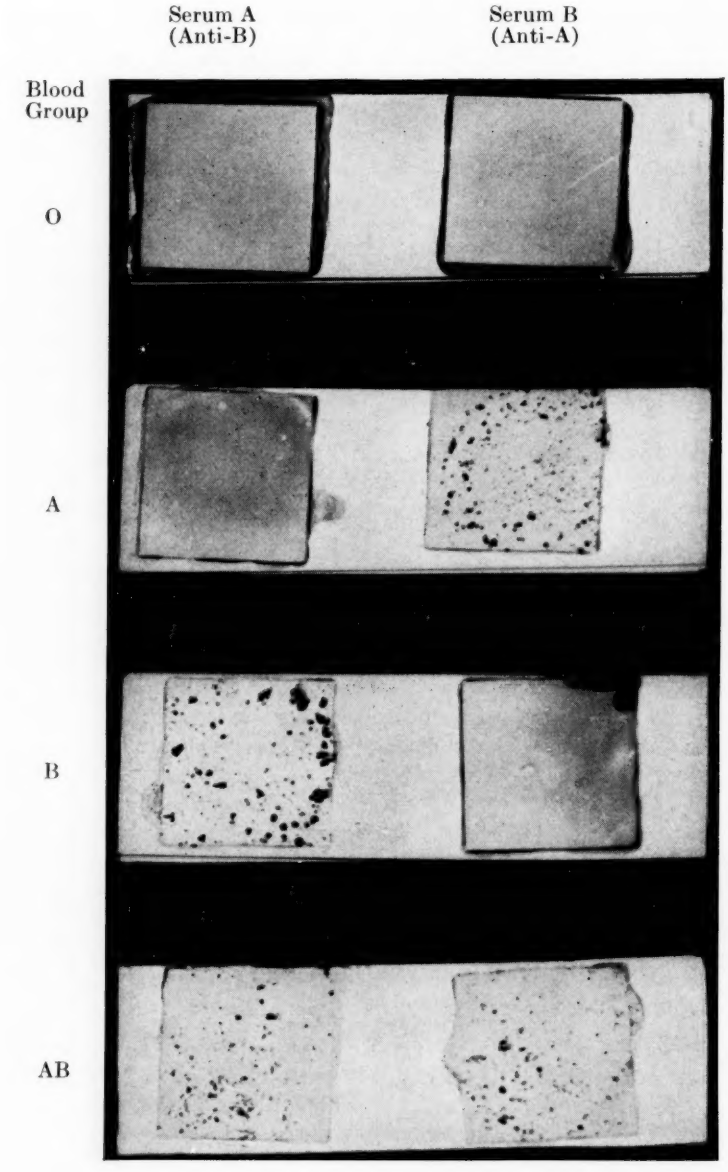

FIG. 2. BLOOD GROUPING ON GLASS SLIDES
(Actual size)

Reproduced by permission from Wiener's "Blood Groups and Blood Transfusion." C. C. Thomas, 1935.

CROSS-MATCHING TESTS

If professional donors are used, their identities and blood groups should be carefully checked by consulting their pass-books. It is advisable to regroup blood stored at the time it is drawn from the "bank" as a protection against technical and clerical errors. If volunteer donors are taken their blood must be tested in the same manner as the patient's.

The question may be raised why it should be necessary to cross-match the bloods of two individuals who belong to the same blood group. The purpose is twofold: (1) as an additional guard against mistakes in technique—experience has repeatedly supported the validity of this reason. (2) to detect any atypical or irregular agglutinins which react independently of the blood groups. These irregular agglutinins are mainly of two sorts. One type is restricted to individuals of groups A and AB and is related to the subgroups. Thus, the serum of individuals of subgroup A_2 or A_2B may occasionally contain an agglutinin acting on blood of subgroup A_1 and A_1B but not on blood of their own subgroup, and, conversely, serum of individuals of subgroups A_1 and A_1B may contain agglutinins acting on A_2 blood. If a patient's serum contains such irregular agglutinins it is important to transfuse him with blood of the homologous subgroup.

The second type of irregular agglutinin is unrelated to the subgroups (or blood groups) and reacts with special agglutinogens. Thus, if a patient of group B with serum containing an irregular agglutinin anti-X is transfused with blood of group B but with cells containing agglutinogen X, a transfusion reaction may result.

The technique of the cross-matching tests is simple.

1. On the left hand side of the slide is placed one drop of the patient's plasma (or serum) and to this is added 1 drop of the donor's cells.

2. On the right hand side of the slide 1 drop each of patient's cells and donor's plasma are mixed (fig. 1).

3. The slide is tilted back and forth for at least 5 minutes; then each mixture is covered with a coverslip and the reactions read under the low power of the microscope.

DETECTION OF MISTAKES IN TECHNIQUE

As has just been mentioned, if the results of the grouping tests on the patient's serum do not correspond to the reactions of the cells, the most likely explanation is that some mistake in technique has been made. Similarly, when two individuals of the same group are not compatible, the possibility of a mistake in technique must be considered. In either case, the first thing to

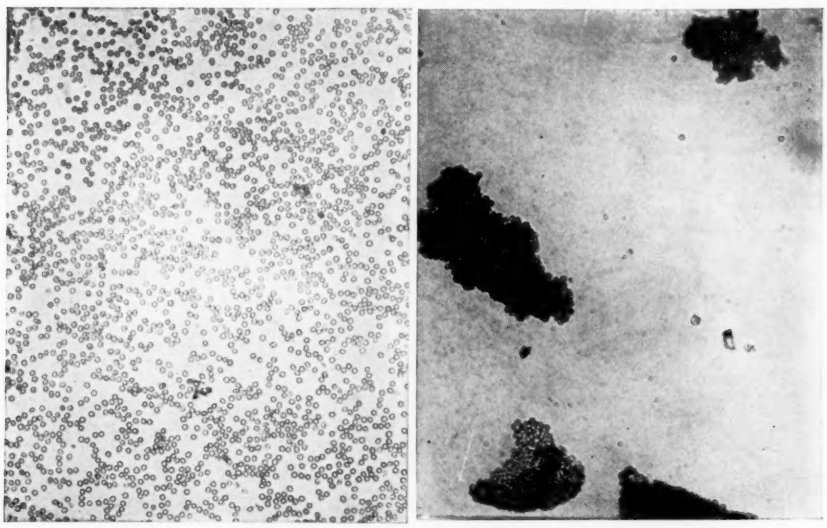

FIG. 3. MICROSCOPIC APPEARANCE OF THE AGGLUTINATION TESTS

Left: Negative reaction—each red blood cell is separate and distinct. *Right.* Positive reaction—the red blood cells are agglutinated together in large clumps.

Reproduced from Wiener's "Blood Groups and Blood Transfusion." C. C. Thomas, 1935.

do is to *check the standard cell suspensions against the standard sera.* If there has been no accidental interchange of these reagents, and if the sera and cells have not deteriorated, the reactions obtained should be clean-cut and should correspond with the labels on the reagents. As a rule, this single check will indicate the source of error and solve the problem. Thus, the standard cell suspensions may have become panagglutinable due

to infection with a particular vibrio or diphtheroid bacillus. Such cells will agglutinate with practically all human sera and therefore will type as "AB." Panagglutinable cells must of course be replaced by fresh suspensions, and in cases where the typing sera do not react properly, these must be discarded.

Rouleaux formation, when very pronounced, may also be mistaken for agglutination, but can readily be recognized, particularly by the practiced observer, merely by examining the cells under the high dry lens. Moreover, this phenomenon is characterized by its ready reversal by slight dilution, or by agitation of the mixture.

Autoagglutination is a phenomenon which may give rise to difficulty in grouping and cross-matching in relatively rare instances. This phenomenon is recognized by the fact that the agglutinins act on all human cells, including the patient's own cells, and the reaction is readily reversed by warming the mixture in an incubator or even on the palm of the hand. For grouping tests, the patient's cells can be freed from autoagglutinins by washing them with warm saline solution, while the serum should be separated from the clot at a low temperature (0°–5°C.).

The presence of irregular agglutinins in the patient's serum should not give rise to difficulties in grouping, since the cells of the patient will give typical reactions. As far as the cross-matching tests are concerned in such cases, if enough donors of the same group as the patient are tested it should be a simple matter to find one compatible with the patient.

THE SUBGROUPS OF GROUP A AND AB

Reference has been made a number of times to the existence of subgroups in group A and group AB and to the rôle played by these subgroups in grouping tests, particularly as a cause of errors in technique. The following brief outline deals with the nature of the subgroups and is given for those readers who may be interested in this question.

The existence of subgroups in group A and group AB depends on the fact that agglutinogen A is not a single entity but includes at least two different sorts of substances, designated by Landsteiner as A_1 and A_2, respectively. Thus, there are two different sorts of group A blood, depending on whether the A agglutinogen is A_1 or A_2, and similarly there are two sorts of AB blood, A_1B and A_2B respectively.

In the serum of group B and group O individuals are agglutinins acting on the agglutinogen A. According to the common concept this isoagglutinin is a single entity designated as anti-A or α. As a matter of fact, it has been found that the so-called α agglutinin is composed of a number of qualitatively different fractions, of which there are two main varieties; (1) anti-A agglutinin proper, reacting with both agglutinogens A_1 and A_2 with approximately equal intensity. (2) agglutinin anti-A_1 or α_1, which reacts with agglutinogen A_1 but practically not at all with agglutinogen A_2.

Different B and O sera contain these two agglutinin fractions in varying quantities and proportions. It is clear, therefore, that two different B sera which react with A_1 cells at approximately the same titer may have different titers when tested against A_2 cells. Thus, one serum, if it contains very much anti-A_1, and very little of the common anti-A agglutinin, might have a titer of 40 for A_1 cells and only a titer of 5 for A_2 cells. On the other hand, another serum which contains a large proportion of common anti-A agglutinin might have a titer of 20 to 40 for A_2 cells even though the titer for A_1 cells is no greater than that of the first serum (40). In extreme instances B serum may react distinctly on A_1 and practically not at all on A_2 cells. In the case of AB blood the danger of using such sera is very great, since the sensitivity of A_2 agglutinogen in this group is greatly diminished and A_2B blood is readily mistaken for group B. This is the reason why every group B serum used for grouping purposes should be titrated against A_2 as well as A_1 cells. Since A_2 makes up approximately $\frac{1}{4}$ to $\frac{1}{5}$ of all group A individuals, it is clear that with weak group B testing sera, 20–25 per cent of group A individuals may be erroneously diagnosed as group O.

Differentiation of A_1 and A_2 blood

The preparation of sera for differentiating group A_1 and A_2 bloods is relatively simple. As has been indicated above, practically all group B sera contain two anti-A agglutinins, namely, the common anti-A agglutinin, which acts on A_1 and A_2 cells, and anti-A_1, which acts only on A_1. By removing the common anti-A agglutinin from such sera, only that agglutinin remains which reacts with A_1 cells, so that in tests made with such a reagent if agglutination occurs the blood must belong to subgroup A_1, and if no agglutination occurs the subgroup is A_2. The reagent is prepared as follows:

1. Blood is drawn from an individual of subgroup A_2, washed three times with saline, packed by centrifuging and the supernatant fluid discarded.

2. A given amount of group B serum is mixed with $\frac{1}{3}$ to $\frac{1}{4}$ as much of the packed, washed A_2 cells.

3. The mixture is allowed to stand for $\frac{1}{2}$ hour at room temperature and centrifuged to separate the serum. It will usually be found that the serum recovered no longer reacts with A_2 cells but still agglutinates A_1 cells distinctly.

Reference has already been made to the possible rôle played by the sub-

groups in transfusion reactions, and some workers advise that only donors of the homologous subgroup be employed for transfusions. In the writer's experience, however, the injection of A_1 blood into A_2 individuals or vice-versa does not give rise to reactions except possibly in the rare instances referred to above where the patient's serum contains irregular isoagglutinins acting on blood of the heterologous subgroup. Therefore, the subgroups can be disregarded except when the patient's serum contains irregular isoagglutinins and here the use of donors of the homologous subgroup is advisable.

DFTERMINATION OF ALCOHOL IN TISSUE AND BODY FLUIDS*

Summary of Practical Procedures for the Pathological Laboratory

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The basis for the determination of the alcohol content in tissues and body fluids is the oxidation of this substance to acetic acid by a suitable oxidizing agent, and determination of the excess reagent either by titration or by colorimetric methods. Potassium permanganate[1], iodine pentoxide[2], alkyl iodide[3], osmic acid[4], and potassium dichromate-sulphuric acid mixture have been employed as oxidizing reagents. The majority of current procedures use the potassium dichromate-sulphuric acid mixture and differ only in the means of separating the alcohol from the body fluids and in the method of measuring the partial reduction of the dichromate. The alcohol may be removed from the specimen by distillation[5,6,7], steam distillation[8], vacuum distillation[9], absorption by a current of purified air[10] or by desiccating the sample in a tightly stoppered flask over the potassium dichromate-sulphuric acid mixture[11,12,13,14].

The extent of the partial reduction of the dichromate has been determined by iodometric titration of the excess dichromate with sodium thiosulphate[1,9,11] by titration with ferrous ammo-

* Received for publication April 27, 1939.

nium sulphate and potassium permanganate[6], or with a mixture of ferrous sulphate and methyl orange[7,13].

The reduction of the potassium dichromate by the alcohol also produces progressive color changes which have been utilized for quantitative colorimetric estimation of the amount of alcohol present. The reduced dichromate solution is compared either with a series of standard solutions (prepared by treating the dichromate reagent with known amounts of alcohol)[5,8,12,15], or with the standard dichromate reagent in the colorimeter using a dark blue filter[14].

We may for convenience divide the methods into two groups, titrimetric and colorimetric. In this paper we shall review only those procedures which because of accuracy and simplicity are applicable for use in the clinical pathology laboratory.

COLORIMETRIC METHODS

A. Method of Heise[5]

Principle. The alcohol contained in a distillate prepared from either blood or urine is treated with the potassium dichromate oxidizing reagent and determined by comparing the partially reduced solution with standards prepared by heating known amounts of alcohol with the dichromate reagent.

Reagents. Potassium dichromate reagent: 333 mgm. of dichromate made to a volume of 100 ml. with 50 per cent sulphuric acid.

Half saturated picric acid containing 10 per cent tartaric acid.

Standard Solutions: Strong Standards—to each of 9 tubes of uniform bore add 3 ml. of the potassium dichromate reagent and the amount of alcohol and water as indicated in column 2 and 3 of table 1. Place tubes in boiling water for four minutes and seal.

Weak standards. To each of fifteen test tubes of uniform bore add 1 ml. of the potassium dichromate reagent and the amount of alcohol and water as indicated in column 2 and 3 of table 2. Place tubes in boiling water for twelve minutes and seal. It is recommended that the standards be prepared fresh every two weeks.

Procedure for urine

To 10 ml. of urine add 10 ml. of half saturated picric acid containing 10 per cent tartaric acid and distill, collecting the first 10 ml. of the distillate. Mix,

and, in a series of three test tubes similiar to those of the standards, add 1 ml. to the first and measured smaller amounts (0.5 ml., 0.2 ml.) in successive tubes. Add water to all but the first tube to bring the total volume to 1 ml. To each of the tubes add 3 ml. of the potassium dichromate reagent and place in boiling

TABLE 1

STRONG STANDARDS

PER CENT ALCOHOL	ALCOHOL BY WEIGHT	WATER
		ml.
0.00		1.00
0.05	0.50 ml. of 0.10 per cent	0.50
0.10	1.00 ml. of 0.10 per cent	
0.12	0.60 ml. of 0.20 per cent	0.40
0.14	0.70 ml. of 0.20 per cent	0.30
0.16	0.80 ml. of 0.20 per cent	0.20
0.18	0.90 ml. of 0.20 per cent	0.10
0.20	1.00 ml. of 0.20 per cent	
0.22	0.73 ml. of 0.30 per cent	0.27

TABLE 2

WEAK STANDARDS

PER CENT ALCOHOL BY WEIGHT	ALCOHOL BY WEIGHT	WATER
		ml.
0.000		2.00
0.005	1.0 ml. of 0.01 per cent	1.00
0.010	2.0 ml. of 0.01 per cent	
0.013	0.52 ml. of 0.05 per cent	1.48
0.016	0.64 ml. of 0.05 per cent	1.36
0.019	0.76 ml. of 0.05 per cent	1.24
0.022	0.88 ml. of 0.05 per cent	1.12
0.025	1.00 ml. of 0.05 per cent	1.00
0.028	1.12 ml. of 0.05 per cent	0.88
0.031	1.24 ml. of 0.05 per cent	0.76
0.034	1.36 ml. of 0.05 per cent	0.64
0.037	1.48 ml. of 0.05 per cent	0.52
0.040	1.60 ml. of 0.05 per cent	0.40

water for 4 minutes. Compare the colors of the unknown tubes with those of the strong standards. The percentage of alcohol by weight is obtained by dividing the reading by the volume of the distillate used.

If the results are too low to be read, use the weak standards. Use 2 ml. and known smaller amounts of the distillate bringing the volume up to a total of 2 ml.

in each case with distilled water. Add 1 ml. of the oxidizing reagent and place the tubes in boiling water for twelve minutes. Make color comparison and base calculation on the volume of distillate used. The following formula may be used.

$$\text{Per cent alcohol by weight in standard} \times \frac{2}{\text{volume of distillate used}} = \text{Per cent alcohol by weight of specimen.}$$

Procedure for blood

To 2 ml. of whole blood, plasma or serum, add 15 ml. of the picric-tartaric acid reagent and distill, collecting the first 10 ml. of the distillate. From this point the procedure is identical with that outlined above for urine, using the weak standards.

The different dilution used in the blood determination is taken into account by multiplying the result obtained by five to give the percentage of alcohol by weight.

According to Heise[5] the color changes produced are so definite that two technicians can consistently check results within 0.01 per cent, when strong standards are used, and 0.002 per cent when the weak standards are used.

The main objections to this method are the time consumed in the distillation and the instability of the standards.

B. Method of Abels[12]

Principle. Abels has combined the colorimetric technique of Heise[5] with the desiccation method of Widmark[11]. Ordinary Erlenmeyer flasks and rolls of filter paper are used in place of the more elaborate flasks proposed by Widmark. A known amount of blood is caught on a small roll of filter paper and subsequently the alcohol which it contains is absorbed into a dichromate-sulphuric acid oxidizing mixture. The alcohol concentration is ascertained by comparing the partially decolorized solution with a series of standards prepared by treating known amounts of alcohol with the oxidizing reagent.

Reagents. 0.33 per cent potassium dichromate in sulphuric acid. Dissolve 333 mgm. of potassium dichromate in 1 ml. of water and dilute to 100 ml. with concentrated sulphuric acid.

0.01 per cent ethyl of alcohol solution. Dilute 10 mgm. of absolute alcohol to 100 ml. with distilled water.

0.05 per cent ethyl alcohol solution. Dilute 50 mgm. of alcohol to 100 ml. with distilled water.

Standard solutions. Fifteen standards are prepared containing 0, 0.05, 0.10, 0.15 ··· 0.70 mgm. To each of fifteen tubes of uniform bore (6″ × ¼″) add 1 ml. of the potassium dichromate reagent and the amounts of alcohol and water as indicated in columns 2, 3, and 4 of table 3. In all cases the total volume is 3 ml. Place the tubes in boiling water for ten minutes and seal. If exposure to light is avoided the standards will keep for at least 2 weeks. It is to be pointed out that these standards have a limited range. The highest concentration of alcohol which can be determined when using a 0.5 ml. sample is 140 mgm. per cent. To keep the number of standards as small as possible a 0.2 ml. sample should be used when the alcohol concentration is above 125 mg. per cent.

TABLE 3

STANDARDS

ALCOHOL CONTAINED IN STANDARD SOLUTION	ML. OF 0.01 PER CENT EtOH	ML. OF 0.05 PER CENT EtOH	WATER
mgm.			*ml.*
0.00			2.00
0.05	0.50		1.50
0.10	1.00		1.00
0.15	1.50		0.50
0.20	2.00		
0.25		0.50	1.50
0.30	0.50	0.50	1.00
0.35	1.00	0.50	0.50
0.40	1.50	0.50	
0.45		0.90	1.10
0.50		1.00	1.00
0.55	0.50	1.00	0.50
0.60	1.00	1.00	
0.65	0.50	1.20	0.30
0.70		1.40	0.60

Procedure for blood

Spread 1 ml. of the dichromate reagent on the bottom of a 50 ml. Erlenmeyer flask, taking care that none of the reagent adheres to the neck of the flask. Absorb 0.5 ml. of blood (when the concentration is above 125 mgm. per cent use 0.2 ml.) onto the roll of filter paper prepared as shown in figure 1. Suspend the roll of filter paper over the oxidizing reagent by inserting the strip of paper attached to the roll between the neck of the flask and the stopper (see fig. 2). It is essential that the flask be tightly stoppered. The flask is either heated for fifteen to thirty minutes in a boiling water bath or in a drying oven at 100°C. After cooling, the stopper and roll of filter paper are removed and 1.7 ml. of water is added to flasks (1.88 ml. is added when 0.2 ml. sample is used), bringing

the total volume to 3 ml.* The diluted solution is poured into a test tube similar to those used for the standards and the color comparison is made.

Calculation.

$$\frac{\text{Amt. of alcohol contained in standard} \times 100}{\text{Volume of sample}} = \text{amount of alcohol per 100 ml. of blood.}$$

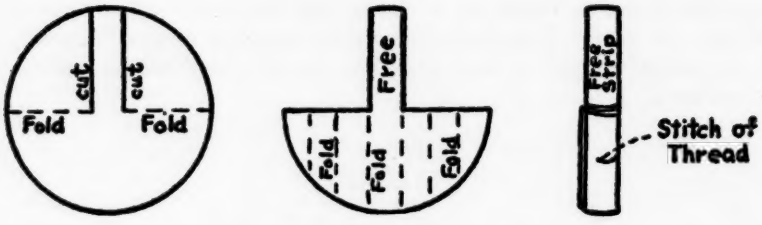

FIG. 1

FIG. 2

Example. Using a 0.5 ml. sample, the unknown matched the standard containing 0.2 mgm. of alcohol thus:

$$\frac{0.2 \times 100}{0.5} = 40 \text{ mgm. per cent.}$$

* The 3 ml. is composed of the dichromate solution (1 ml.), the added water (1.7 or 1.9 ml.) and the water derived from the sample of blood (0.3 ml. from the 0.5 ml. specimen or 0.12 from 0.2 ml. specimen).

This method gives over 90 per cent recovery, which is sufficiently accurate for clinical diagnosis of alcoholism. The method is simple and does not require special apparatus. However the same objection applies to this method as to that of Heise[5] namely that the prepared standards are somewhat unstable and must be replaced every two weeks.

Abels[12] also describes a micro method based on the same principle. The alcohol is determined in 0.05 ml. of blood. Smaller quantities of reagents are used and the oxidation is carried out in a special Erlenmeyer flask with a side arm whose bore is identical with that of the standard tubes. After the absorption and oxidation of the alcohol the flask is tipped on its side allowing contents to flow into the side arm and color comparison is made with the prepared standards. This method is to be recommended where only minute quantities of blood are available for analysis. For detailed description of the micro procedure see the original article.

C. Method of Sheftel[14]

Principle. This is essentially a modification of Abels'[12] method. The partially decolorized potassium dichromate solution is compared with the dichromate reagent in a colorimeter, using a blue filter. The filter facilitates matching colors as the partially reduced dichromate has a blue-greenish tint due to the presence of chromic sulphate.

Apparatus. 50 ml. Pyrex flask; rubber stopper in which a monel or nichrome wire clip is inserted to keep filter paper in place; filter paper strips of medium thickness 1¼″ x 2″; a special blue filter for colorimeter,† and a 0.2 ml. pipette.

Reagent: 0.4262 per cent potassium dichromate in 50 per cent (by volume) sulphuric acid. 1 ml. of this reagent is completely reduced by 1 mgm. of ethyl alcohol.

Procedure for blood and urine

Spread 1 ml. of the dichromate reagent on the bottom of the flask. Roll the filter paper and insert it into the clip attached to the stopper and absorb onto the filter paper 0.2 ml. of either blood or urine. Insert the cork but not too tightly into the neck of the Erlenmeyer flask. The neck is carefully heated on all sides over a bunsen flame and flask is tightly stoppered and placed in boiling water for fifteen minutes. The flask is removed from the boiling water, the cork and roll of filter paper are carefully removed, and the flask is cooled under tap water. To the cooled flask add 4.3 ml. of distilled water.

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† Lantern blue glass filter No. 554, manufactured by the MacGregor Instrument Co. of Needham, Mass.

The standard is prepared by adding 10 ml. of distilled water to 2.5 ml. of the dichromate reagent (dilution 1:5). 1 ml. of the unknown is diluted to 5.4 ml.‡ Approximately 0.1 ml. of blood remains on the filter paper as unevaporated material. The difference in the dilution is according to Sheftel a necessary correction for the absorption of light in the colorimeter by chromic sulphate. Set the unknown at ten and make color match by adjusting the standard solution in any micro-colorimeter using a blue filter.

Calculation.

$$\left(1 - \frac{R}{10}\right) \times 500 = \text{mgm. alcohol per 100 ml.}$$

R = reading of standard.

Example. R—7.4; then: $(1 - \frac{7.4}{10}) \times 500 = 130$ mgm. per 100 ml.

Sheftel has obtained practically complete recovery of alcohol. In experiments in which 100 mg. of alcohol were added to blood and 200 mgm. to urine Sheftel obtained an average recovery of 103 per cent for blood and 99 per cent for urine.

TITRATION METHODS

A. Method of Newman[9]

Principle. The alcohol is removed from the specimen of either blood or urine by vacuum distillation and bubbled through a potassium dichromate-sulphuric acid oxidizing mixture. The excess dichromate is determined by iodometric titration with sodium thiosulphate.

Reagents. Oxidizing solution. Equal parts of concentrated sulphuric acid and approximately 0.1N potassium dichromate (4.9 grams of potassium dichromate made up to a liter with distilled water). Acid should be added to the dichromate slowly and with cooling under tap water.

0.1N Stock thiosulphate solution. Dissolve 24.82 grams of Reagent Quality sodium thiosulphate in water. Add 2 ml. of 10 per cent sodium hydroxide and make up to one liter with distilled water.

0.025N sodium thiosulphate solution. To 25 ml. of the 0.1N stock thiosulphate solution add sufficient distilled water to make up to a volume of 100 ml.

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‡ 1 ml. of dichromate, 4.3 ml. of water and 0.1 ml. of water derived from the sample.

20 per cent potassium iodide solution.

1.0 per cent starch solution.

Where the highest degree of accuracy that the method is capable of yielding is desired the thiosulphate solutions should be standardized. We have found, however, that for clinical purposes the solutions can be prepared from a good grade of thiosulphate by carefully weighting out the required amount. These solutions checked well with the standardized solutions. The 0.025N thiosulphate solution should be prepared fresh from the stock solution. 1 ml. of 0.025 N sodium thiosulphate solution is equivalent to 0.2875 mgm. of alcohol.

Procedure

Sufficient anhydrous sodium sulphate is introduced into a 50 ml. Erlenmeyer flask to thinly cover the bottom. Onto this is delivered exactly 1.0 ml. of the fluid to be analyzed. The flask is then tightly stoppered until ready for the

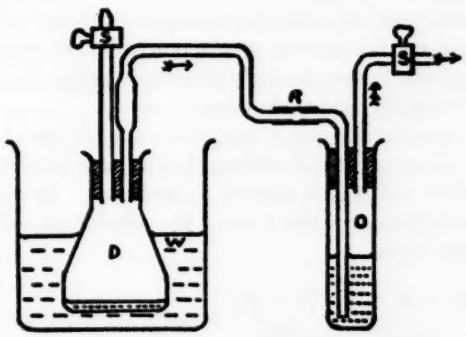

FIG. 3

determination. In analyzing the sample, the stopper of the flask is replaced by one bearing an inlet tube closed by a stopcock, and an outlet tube, for connection by means of a short rubber tube with the inlet of the oxidizing tube as shown in figure 3. The latter is a test tube, $\frac{3}{4}$ x 5 inches containing 5 ml. of the oxidizing solution. It is fitted with a two-hole stopper which bears the inlet tube. This is connected with the outlet tube of the Erlenmeyer flask and dips well into the oxidizing mixture. It has a perforated bulb bottom to minimize the size of the bubbles produced on distillation. The outlet tube connects through a stopcock to an efficient water or oil suction pump. The Erlenmeyer flask containing the sample is placed in a water bath kept at 40 to 45°C., and a vacuum obtained by suction is maintained for fifteen minutes. The stopcock to the pump is closed, and the vacuum broken by slowly opening the stopcock on the inlet to the Erlenmeyer flask. The oxidizing tube is removed with its stopper and the contents carefully washed into a 300 ml. Erlenmeyer flask.

The volume is made up to at least 100 ml. with distilled water; 10 ml. of 20 per cent potassium iodide are added, and titration with 0.025N sodium thiosulphate carried out using starch as the indicator. A clearer end-point is obtained if the starch is not added until near the end, when the color changes from yellow to yellow-green.

The time required for each determination is about twenty minutes but by using two sets of apparatus it is possible for a single operator to run six or seven analyses an hour.

The chemical reactions which take place are as follows:

$$2K_2Cr_2O_7 + 8H_2SO_4 + 3C_2H_5OH = 2Cr_2(SO_4)_3 + 2K_2SO_4 + 3CH_3COOH + 11H_2O$$

$$K_2Cr_2O_7 + 6KI + 7H_2SO_4 = 4K_2SO_4 + Cr_2(SO_4)_3 + 7H_2O + 3I_2$$

$$2Na_2S_2O_3 + I_2 = 2NaI + Na_2S_4O_6$$

Blank determination. A blank determination is run, using distilled water in place of the unknown and following the same procedure as outlined above. This controls oxidizable matter present in the reagents. In case of blood a blank is run on blood known to contain no added alcohol since blood itself contains a small amount of volatile oxidizable substances.

Calculation. A is the volume of 0.025N sodium thiosulphate used to titrate blank, B is the volume of 0.025N sodium thiosulphate used to titrate unknown. $(A-B) \times 0.2875$ = amount of alcohol in specimen. In case of 1 ml. samples the product is multiplied by 100 to give the number of milligrams of alcohol per 100 ml. of the fluid.

$$\frac{(A - B) \times 0.2875 \times 100}{\text{Volume of sample}} = \text{mgm. alcohol per cent.}$$

The solutions described adequately cover the range from 20 to 250 mgm. per 100 ml. using 1.0 ml. samples. By varying the size of the sample and concentration of the oxidizing mixture, concentrations of alcohol outside of these limits may be determined.

With this method the author states that in specimens containing 1 mgm. of alcohol, or more, the probable error does not exceed 1.5 per cent.

The chief drawback of this method is the difficulty encountered by some technicians in recognizing the starch-iodine end-point when using 0.025N sodium thiosulphate. In our experience we have found that with a little practice the end-point can readily be determined.

B. Method of Harger[7]

Principle. An aliquot of a distillate prepared from diluted urine or a protein free filtrate of blood is treated with potassium dichromate-sulphuric acid mixture and excess of the reagent is

titrated with a mixture of ferrous sulphate and methyl orange. Methyl orange is destroyed by the dichromate reagent, but as soon as all the reagent is reduced the solution turns pink indicating the end point.

Reagents: Concentrated Sulphuric acid. A good grade of C.P. acid containing very little reducing substances.

Sulphuric acid (62 per cent), pour one volume of concentrated C.P. sulphuric acid slowly and carefully into an equal volume of water and cool.

Methyl orange (0.1 per cent), dissolve 1 gram of methyl orange in one liter of approximately N/40 NaOH and filter. This will keep indefinitely.

Ferrous sulphate (20 per cent), dissolve 50 grams of $FeSO_4 \cdot 7H_2O$ in 150 ml. of water. Add 30 ml. of concentrated sulphuric acid and dilute to 250 ml. In a stoppered flask this solution will undergo little oxidation for one year.

Red reducing fluid. Place in a flask 35 ml. of the 62 per cent sulphuric acid. Add to this 15 ml. of the methyl orange solution and 1 ml. of the ferrous sulphate, shaking the flask during the addition. Mix well and cool to room temperature. This solution deteriorates slowly but will keep for three or four days, being more rapidly decomposed in warm weather than in cold. About 2.5 ml. of this solution are required for each ml. of the standard dichromate solution.

Standard dichromate solution, 0.0434 N. Dissolve 2.129 grams of C.P. potassium dichromate in water and make up to one liter. One milliliter of this solution is equivalent to 0.5 mgm. of ethyl alcohol (ethanol) or 0.232 mgm. of methyl alcohol (methanol). The dichromate may be standardized against a weighed amount of alcohol or in the manner described in standard works on quantitative analysis. Harger states that a good grade of C.P. analyzed potassium dichromate (Baker and Adamson or J. T. Baker) is sufficiently pure for use as a primary standard.

Procedure

Removal of alcohol from tissues and biological fluids. Tissue. The material is finely hashed, mixed with an equivalent weight of water and acidified with a little tartaric acid. It is then steam distilled until the volume of the distillate equals the weight of the tissue taken. One milliliter of the distillate is diluted to 50 ml. and 5 ml. of the diluted solution, representing 0.1 gram of tissue, is taken for analysis. Where tissue alcohol is less than 0.05 per cent, dilution of distillate should be less than 1:50. To correct for the dilution of distillate the following formula may be used:

Dilution $\times$ Ml. of diluted solution = Equivalence in gram of tissue

Example. 1 ml. of distillate is diluted to 20 ml. and 5 ml. of diluted solution is taken for analysis then: $1/20 \times 5 = 0.25$ gram of tissue. If alcohol in tissue is above 0.5 per cent use less than 5 ml. of distillate (1:50 dilution) and add enough

water to make up to 5 ml. To correct for such dilution use the following formula:

$$\frac{0.1 \times \text{ml. of diluted solution taken}}{5} = \text{equivalence to gram of tissue taken for analysis.}$$

Blood. Prepare a tungstic acid protein-free filtrate of blood by the Folin-Wu method. In a 125 ml. pyrex distilling flask place 5 ml. of the filtrate, 20 ml. of water and some glass beads. Connect the flask to a small vertical condenser, the lower end of which extends well into a Folin receiving tube graduated to 25 ml. Heat the distilling flask directly over a small flame from a micro burner. Distill until about 12 ml. of liquid is collected. This takes about five minutes. Rinse the condenser tube with a little water and collect washings in the receiving tube. Dilute contents of receiving tube up to 25 ml. with distilled water. Five milliliters of this distillate, representing 0.1 ml. of blood, are used for analysis. If alcohol is above 0.5 per cent the procedure is modified as described under *tissues*. If distillate is to be kept some hours before analysis it should be acidified with a few drops of concentrated sulphuric acid.

Urine. Dilute 1:10 and follow identical procedure as outlined for the protein-free filtrate of blood.

Analysis of distillates

In a reaction tube place 5 ml. of the solution of alcohol from the distillation of blood, urine or tissue, which should not contain more than 0.5 mgm. of ethanol or 0.23 mgm. of methanol. Add 1 ml. (where the sample analyzed contains less than 0.05 mgm. ethanol, the accuracy is improved by using less dichromate, 0.5 to 0.2 ml., and reducing the amount of ferrous sulphate in the red titration fluid to 0.5 ml. or 0.2) of the standard dichromate and then 5 ml. of concentrated sulphuric acid. Mix well by means of a stirring rod and allow to stand for 10 minutes. Cool in water to room temperature and titrate the excess of dichromate with the red reducing fluid. The first permanent pink color is the end-point. In the same manner run a blank on 5 ml. of distilled water. Since sulphuric acid usually contains a trace of reducing material, the correct figure for expressing the relationship of the red reducing fluid in terms of dichromate is obtained by adding to one of the tubes which has been titrated to the end-point an additional 1 ml. of the standard dichromate and again titrating to the end-point.

Calculation. U, titration figure for unknown; W, titration figure for distilled water; B, titration figure for extra milliliter of dichromate; Q, quantity of blood, urine or tissue represented by aliquot analyzed then,

$$\frac{\frac{W - U}{B} \times 0.5}{Q} = \text{mgm. alcohol per ml. of blood, urine or gram of tissue.}$$

For methanol use 0.23 instead of 0.5.

Example: Titration of unknown = 1.9 ml. Titration of distilled water (blank) = 2.4. Titration of extra milliliter of dichromate = 2.5. Quantity of tissue taken for analysis = 0.25.

Then:

$$\frac{\frac{2.4 \times 1.9}{2.5} \times 0.5}{0.25} = 1.00 \text{ mgm. alcohol per gram of tissue.}$$

The introduction of the mixture of ferrous sulphate and methyl orange with its sharp end-point makes the titration of the excess dichromate quite simple and accurate. A drawback to this method is the time involved in the initial distillation. Harger's data show good recovery with samples containing 0.01 to 0.4 mg. of alcohol. The author describes a device for stirring with a current of air during the titration. The reader is referred to the original paper for further details.

C. Method of Cavett[13]

Principle. The Widmark[11] type of distillation (desiccation of the specimen over the oxidizing reagent) is combined with the titration method of Harger[7] using the ferrous sulphate-methyl orange reducing fluid.

Reagents. Standard dichromate solution. Dissolve 0.4258 gram of C.P. potassium dichromate in water and make up to one liter. One milliliter of this solution is equivalent to 0.1 mgm. of alcohol.

Sulphuric acid (62 per cent), methyl orange (0.1 per cent), ferrous sulphate (20 per cent), red reducing fluid. The last four reagents are prepared as outlined under Harger's[7] method.

Procedure

Place 5 ml. of the standard dichromate solution in the special 50 ml. Erlenmeyer flask (fig. 4) and add 50 ml. of concentrated sulphuric acid. Into the small cup attached to the stopper place either 0.1 or 0.2 ml. of fluid to be analyzed and carefully insert the stopper in the flask. Place flask in the oven at 70°C. for 2 hours, or allow it to stand at room temperature for 12 hours (over night). Remove the stopper and cup and titrate the contents of the flask with the red reducing fluid. The yellow solution changes to a definite pink at the end-point. A blank determination is run simultaneously. After titrating one of the flasks and obtaining the required end-point, add an additional 5 ml. of the dichromate reagent and titrate with the red reducing fluid. This will give the relationship of the reducing fluid to the standard dichromate.

FIG. 4

Calculation: Since 5 ml. of the dichromate is completely reduced by 0.5 mgm. of alcohol, twice the amount of the red reducing fluid required to titrate the additional 5 ml. of dichromate is equivalent to 1 mg. of alcohol. Then:

$$\frac{B - A}{2C} = \text{mgm. alcohol present in sample,}$$

or:

$$\frac{B - A}{2C} \times \frac{100}{Q} = \text{mgm. alcohol in 100 ml. of fluid}$$

where: A = ml. of reducing fluid required for unknown, B = ml. of reducing fluid required for blank, C = ml. of reducing fluid required for extra added 5 ml. of dichromate, Q = quantity of fluid taken for analysis.

REFERENCES

(1) FRIEDEMANN, T. E., AND RITCHIE, E. B.: A method for the determination of ethyl alcohol. Proc. Soc. Exper. Biol. and Med., **30:** 451, 1933.

(2) HAGGARD, H. W., AND GREENBERG, L. A.: The quantitative determination of ethyl alcohol in air, blood, and urine by means of iodine pentoxide. J. Pharmacol. and Exp. Therap., **52:** 137, 1934.

(3) GETTLER, A. O., NIEDERL, J. B., AND BENEDETTI-PILCHER, A. A.: The isolation of pure anhydrous ethyl alcohol from non-alcoholic human and animal tissues. J. Am. Chem. Soc., **54:** 1476, 1932.

(4) Hiramatsu, T.: New method of determination of alcohol in blood. Sei-i-Kwai Med. J., **48:** 21, 1929.
(5) Heise, H. A.: The specificity of the test for alcohol in body fluids. Am. J. Clin. Path., **4:** 182, 1934.
(6) Nicloux, M. M., Le Breton, E., and Dontcheff, A.: Microdosage, dans le sang et les tissus, de quantites d'alcoöl ethylique. Bull. Soc. Chem. Biol., **16:** 1314, 1934.
(7) Harger, R. N.: A simple micromethod for the determination of alcohol in blood. J. Lab. and Clin. Med., **20:** 746, 1935.
(8) Bogen, E.: Drunkenness. A quantitative study of acute alcoholic intoxication. J. Am. Med. Assoc., **89:** 1508, 1927.
(9) Newman, H.: The determination of ethyl alcohol in body fluids. J. Pharmacol. and Exper. Therap., **56:** 278, 1936.
(10) Bogen, E.: Drunkenness. A quantitative study of acute alcoholic intoxication. Am. J. Med. Sci., **176:** 153, 1928.
(11) Widmark, E. M. P.: Eine Mikromethode zur Bestimmung von Athylalkohol im Blut. Biochem. Z., **131:** 473, 1922.
(12) Abels, J. C.: A simple method for the determination of ethyl alcohol in blood. Proc. Soc. Biol. and Med., **34:** 346, 1936.
(13) Cavett, J. W.: The determination of alcohol in blood and other body fluids. J. Lab. and Clin. Med., **23:** 543, 1938.
(14) Sheftel, A. G.: A simple colorometric method for determination of alcohol concentration in urine and blood. J. Lab. and Clin. Med., **23:** 534, 1938.
(15) Nicloux, M. M.: Dosage de l'alcoöl ethylique dans des solutions ou cet alcoöl est dilué dans proportions comprise entre 1/500 et 1/3000. Compte, rend. Soc. de Biol., **48:** 481, 1896.

THE MEDIUM OF WILSON AND BLAIR FOR THE DETERMINATION OF CLOSTRIDIUM WELCHII*

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In 1924 Wilson and Blair described a medium containing sodium sulfite and ferric chloride to be used for estimating the number of anaerobes in water. The medium is made as follows: To 100 cc. of 3 per cent nutrient glucose agar add 10 cc. of freshly prepared 20 per cent solution of sodium sulfite and 1 cc. of 8 per

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cent solution of ferric chloride. To 20 cc. of this medium is added 20 cc. of the water to be tested and the medium is poured into a plate. When the agar has solidified another equal layer of agar and sterile water is poured on top of the first to act as a seal.

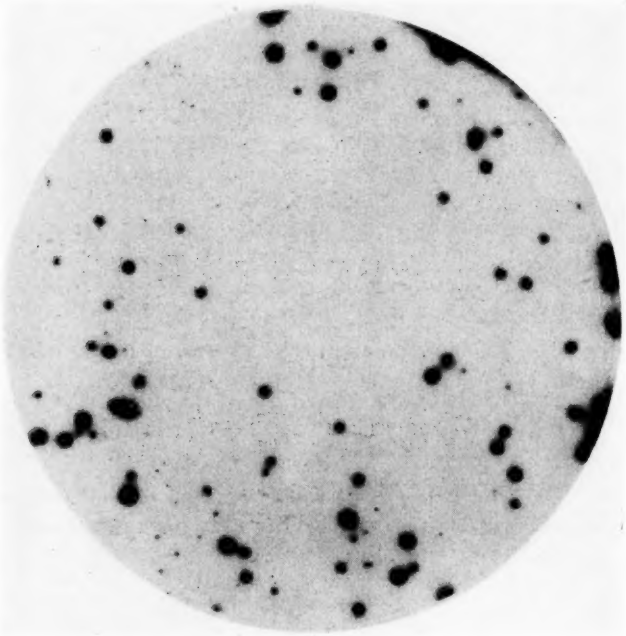

FIG. 1. CLOSTRIDIUM WELCHII ON WILSON AND BLAIR MEDIUM AFTER INCUBATION FOR SIXTEEN HOURS (8/9 ACTUAL SIZE)

The authors stated that several anaerobes would produce black colonies in this medium and mentioned Clostridium welchii, Clostridium fallax, and Clostridium oedematiens.

In 1926 Greer used the medium of Wilson and Blair to estimate the number of anaerobes in sewage. He reported that of 160 black colonies picked from this medium 153 were Clostridium welchii and seven were Clostridium sporogenes.

Since 1930 I have used this medium for detection of Clostridium welchii in clinical material, and consider it a very valuable addi-

tion to the methods available to the clinical bacteriologist. Upon this medium Clostridium welchii will develop a black color in six or seven hours with a moderate amount of inoculum. In materials in which gram-positive organisms resembling Clostridium welchii can be found in stained smears it is necessary to make considerable dilution of the material in order to get isolated colonies. Figure 1 shows the appearance of Clostridium welchii in Wilson and Blair's medium after sixteen hours' incubation. The medium is almost completely inhibitory to gram-positive cocci and partly so to gram-negative bacilli of the intestinal group. In my experience with clinical material, blackening of this medium within six or eight hours and the appearance of isolated colonies which have black zones measuring 2 to 3 mm. in diameter, within sixteen hours have invariably denoted the presence of Clostridium welchii.

The only changes which I have made in Wilson and Blair's original formula for the medium have been made with the idea of adapting it for clinical use. Instead of nutrient agar, beef infusion agar has been used, with the idea that growth would be more rapid. The concentration of agar has been made 2 per cent instead of 3 per cent and 0.5 per cent glucose has been used. This medium has been bottled in 100 cc. amounts and about 3 grams of marble chips has been added before autoclaving. The reaction has been set at pH 7.0, using bromthymol blue indicator. For use, a bottle of the agar has been melted in boiling water, and the sodium sulfite and ferric chloride added when the temperature of the medium has cooled to 45°C. Suitable dilutions of the material to be tested were put in petri dishes and about 20 cc. of the prepared medium was poured in and mixed by rotation. When the inoculated layer had hardened an equal amount of the same medium was poured on top as a seal.

Recently, Robinson and Stovall have used the same principle as Wilson and Blair for the detection of Clostridium welchii, but have substituted milk for the agar. The concentrations of sodium sulfite and ferric chloride were unchanged. They stated that this medium is highly specific for Clostridium welchii. It should be very satisfactory for the qualitative determination of

Clostridium welchii but can hardly give the quantitative information which is afforded by a plating medium. In addition, it appears that the results may be read somewhat sooner on the agar medium than on the milk medium, judging by the figures which these authors have given.

REFERENCES

(1) WILSON, W. J., AND BLAIR, E. MAUD M'V.: The application of a sulphite-glucose-iron agar medium to the quantitative estimation of *B. welchii* and other reducing bacteria in water supplies. J. Path. & Bact., **27:** 119–121, 1924.

(2) GREER, F. E.: Anaerobes in sewage. Am. J. Pub. Health, **16:** 577–578 (June), 1926.

(3) ROBINSON, C. LENORE, AND STOVALL, W. D.: A clinical bacteriological test for the recognition of C. welchii in wounds. Am. J. Clin. Path. (Tech. suppl.), **9:** 27–32 (Jan.), 1939.

A METHOD FOR THE EXAMINATION OF THE CELLULAR ELEMENTS OF BODY FLUIDS

A COLLODION SAC FOR CONCENTRATION AND PAARFFIN-EMBEDDING*

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We have found that by the use of an inexpensive, easily made, semi-permeable collodion sac the cellular elements of fluid materials can be readily concentrated, fixed, and embedded in paraffin. There is no danger by this method of loss or distortion of the formed material to be examined.

PREPARATION OF THE SAC

A 15 cc. conical centrifuge tube is filled to the brim with collodion, U.S.P. It is most important that the tube be dry and chemically clean, and that the collodion be perfectly fresh. The collodion is then poured back into the stock

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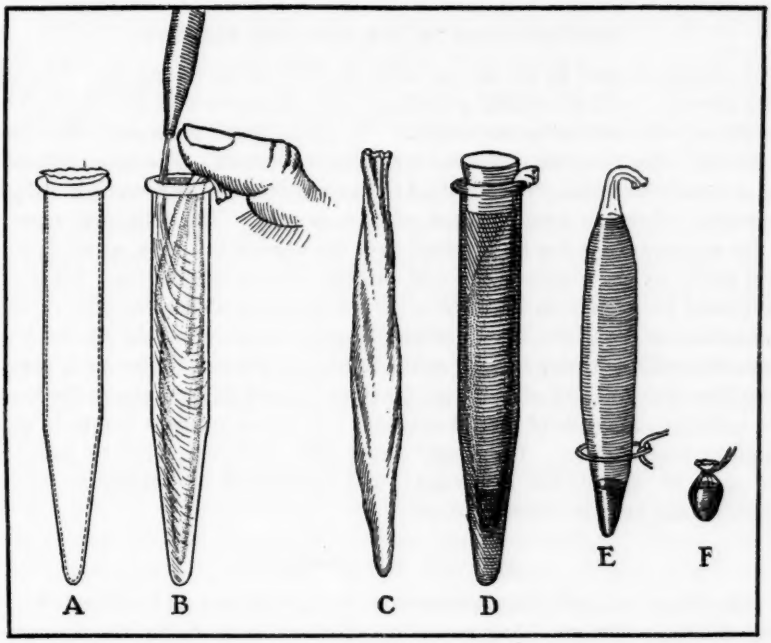
bottle with a rotary motion, so that the lip of the tube is uniformly and completely covered with it. The tube is clamped in an inverted position and allowed to drain for ten minutes; a current of air passed into the mouth will hasten the evaporation of the ether from the collodion. At the end of this time



METHOD OF PREPARING AND USING COLLODION SAC

A. Edges of the collodion sac curled from the lip of the centrifuge tube toward the center.

B and C. Separation and removal of the sac accomplished by traction and the passage of a thin stream of water between it and the inner wall of the tube.

D. Sac suspended in centrifuge tube after centrifugalization of the fluid to be examined. Note the sediment at the bottom of the sac. Note also that the space around the sac is completely filled with fluid.

E. Sac after removal from the tube. Note the tie ready to be fastened above the level of the sediment.

F. Sac with its content of sediment ready for immediate fixation.

the inside of the tube is rinsed with water and the collodion sac is removed: The edges of the collodion sac which are adherent to the lip of the tube are carefully curled toward the center, and, after separation has begun, a thin stream of water from a wash bottle is allowed to flow between the sac and the wall of

the tube. The sac can then be easily lifted out by gentle traction. After removal it is tested for leaks by filling it with water. It is transferred to a storage bottle containing a 1:10,000 aqueous solution of merthiolate, from which it is not removed until just before it is to be used. A large supply of the collodion sacs can be prepared at one time and kept for several months.

CONCENTRATION OF THE CELLULAR ELEMENTS

A pipette is used to fill the sac with the fluid to be examined. The sac is then placed in a 15 cc. conical centrifuge tube, the inner wall of which has been moistened with normal saline solution. If a thin, moist glass rod is first placed in the tube, the insertion of the sac is greatly facilitated. The space around the sac is completely filled with the fluid to be examined if it is present in sufficient quantity; otherwise normal saline solution is used. The collapsed, open end of the sac meantime has been folded over the edge of the tube, where it is now held firmly by the insertion of a cork stopper of appropriate size. Little or no air should be present in the tube after the insertion of the stopper. Centrifugalization is then done for the proper length of time; when the process is complete, the sediment may be seen at the bottom of the sac. The sac is removed from the tube and tied off with no. 20 thread about 0.3 cm. above the level of the sediment, the ends of the thread being cut to a convenient length to permit handling with forceps. The empty upper part of the sac is cut off just above the point of the tie, and discarded. The material is now ready for fixation, which should be done without delay.

FIXATION AND EMBEDDING

The intact sac with its contents may be fixed in any of the commonly employed fixing solutions. Any one of the standard methods for dehydration and paraffin-embedding of tissue may be employed, provided that it does not require the use of acetone or other collodion solvents.

COMMENT

To date we have used this method for such fluid materials as ascitic, pericardial, and pleural fluid, suspended bone marrow cells, and exudates of various sorts. We have found it entirely satisfactory, and it seems quite possible that other uses will develop as it continues to be employed.

LABORATORY DIAGNOSTIC INDEX*

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A card index of surgical and autopsy diagnoses is an indispensable part of every orderly, efficient laboratory. The indexing of these diagnoses in this hospital has been most satisfactorily accomplished by placing the principle burden upon the pathologist who makes the diagnosis and who alone has a definite idea as to the anatomy, etiology and nature of the disease. To execute this with the greatest ease to the pathologist, he may put a number after each final diagnosis which is the number of a card in the index file. A helper can then find the proper card and write on it the autopsy or surgical laboratory number of the case. The same file may be used for both, with autopsy diagnoses on cards of one color and surgical diagnoses on cards of another color.

The system of numbers used in the Standard Classified Nomenclature of Disease forms a most desirable basis. A copy of the system should be near the pathologist's desk (New York, The Commonwealth Fund, 1935). We have abbreviated to a considerable extent this detailed classification so as to make it possible to have all of the diagnoses and numbers on the two sides of a card board from a 14 x 17 X-ray film box. Thus the numbers can be found quickly and added to each diagnosis as it is being made. In time as more numbers are added to the system a more complicated but easily handled key to the index may be set up on the pathologist's desk.

The Standard Classified Nomenclature depends firstly on topography, each region having a system number and any one component part has two division numbers. Thus each anatomic structure has a number of three digits, eg. 6 = digestive system, 4 = stomach, and 2 = fundus, or 642 = fundus of stomach. For abbreviation we use 640 = stomach as a whole. Secondly the classification depends on etiology. Category 8 = neoplasm,

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1 = origin in connective tissue, 2 = benign fat cell, or (640–812) = lipoma of the stomach. For abbreviation we use (640–8–lipoma). A laboratory assistant then finds the index card 640–8 and writes on it the surgical or autopsy laboratory number followed by (lipoma).

The degree of abbreviation of the Standard Classified Nomenclature of Disease is entirely arbitrary and in time we may re-classify certain single entries in the index into finer divisions. At present it is more expedient to add some anatomic, diagnostic, or etiologic appendage to certain numbers which may represent one organ rarely found diseased. For instance, the thymus has one card (no. 830) and to have any more would unnecessarily complicate and enlarge the file. All diseases of this gland are filed on this one card and the name of the disease is written after the laboratory number. It is admitted that the use of the entire index system of the Standard Classified Nomenclature with some minor alterations and additions would be ideal if a trained helper in the laboratory can be completely trusted to do the work.

We classify all neoplasms according to this system and also on a simplified histologic basis. The latter index is not necessary but does not add significantly to the work and may simplify the group study of neoplasms by students or physicians in the laboratory. Of course, this double index may be used for infectious, traumatic, congenital, and other types of diseases.

I have mimeographed copies of the diagnoses and numbers used at present in this hospital so that if the reader wishes to start on the same basis and use the same system, he can paste these mimeographed sheets on the 14 x 17 card board. He may then add to or shorten the classification as new problems arise.

ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

BLOOD AMYLASE OR DIASTASE DETERMINATIONS

To the Editor: Do you have the method for doing Blood amylase or diastase, according to the method used by Myers and Killian, using the Myers-Bailey method for blood sugar? Mention was made of it in one of the Technical Supplements, of the American Journal of Clinical Pathology, some months ago, but the complete method was not given. The Journal of Biolog. Chem. for

January 1933 does not publish the method, and I am unable to get a copy of the Journal for 1917. The clinical laboratory books do not seem to think it important enough to publish.

M. L.

Answer: The method described by Myers and Killian (J. Biol. Chem., **29:** 179, 1917) is as follows: "Two 2 cc. samples of oxalated blood are taken, one being employed as a control. The control tube is made up to 10 cc. with distilled water, and the tube to be employed for the test to 9 cc. Both tubes (cylindrical centrifuge tubes) are now placed in a water bath at 40°C. As soon as the contents of the tubes have been brought to this temperature, 1 cc. of 1 per cent soluble starch is added to the second tube, the contents are mixed, and incubation is then carried out for exactly 15 minutes at 40°C. After the incubation has been completed, about 1 gram of dry picric acid is at once added to each tube and the mixtures are stirred. When the proteins are precipitated, the tubes are centrifuged and the yellow supernatant fluid is filtered. The sugar in 3 cc. portions of the filtrates is now estimated according to the technique of Myers and Bailey (J. Biol. Chem., **24:** 147, 1916). Correction is made for the sugar originally present in the blood (with the aid of the control) and for the slight reducing action of the soluble starch. The results have been recorded in terms of the percentage of soluble starch (10 mgm.) transformed to reducing sugars (calculated as glucose) by the 2 cc. of blood employed.

"It is believed that 10 mgm. of starch furnish a sufficient substrate for all ordinary conditions with human blood (except possibly in very severe diabetes), since practically identical results have been obtained with amounts up to 40 mgm. Glycogen offers no advantage over soluble starch.

"The scheme we have followed in recording our results is an arbitrary one, but the figures obtained, ranging from 15 to 75, are satisfactory for expressing the variations in the diastatic activity of human blood. If it is desired to express the data in mg. of reducing sugar formed per 100 cc. of blood, this may readily be done by multiplying the above figures by 5."

The editor acknowledges dereliction in not giving the complete method of Somogyi in answering a query in the March 1939 number of the Supplement, on the same subject, and herewith publishes the procedure as given out in mimeograph form by Elman, Hoagland, Hemplemann and Brown of Washington University School of Medicine at their booth in the Scientific Exhibit of American Medical Association this year. This is an abstract of Somogyi's paper published in the J. Biol. Chem. **125:** 399, 1938.

SOMOGYI'S SACCHAROGENIC METHOD FOR THE ESTIMATION OF AMYLASE (DIASTASE) AS USED AT THE BARNES HOSPITAL

The *amylase* method depends on the determination of the reducing sugars formed by the action of amylase in blood serum (plasma) on starch.

REAGENTS NEEDED

Starch Paste. U.S.P. corn-starch or pure rice starch is washed as follows: 100 grams of starch are suspended and frequently agitated for about an hour in 1 liter of approximately 0.01 N HCl. After sedimentation the acid is poured off and the starch is stirred up in 1 liter of approximately 0.05 per cent NaCl solution. After sedimentation and decantation, washing with salt solution is repeated once more; then the starch is spread out and allowed to dry in air.

One liter of paste is prepared by thoroughly grinding in a mortar 15 grams of washed dried starch with 50 cc. of water, while 900 cc. of water are heated to boiling. The ground starch suspension is transferred into the hot water with vigorous agitation, 50 cc. of water being used to rinse the mortar. After boiling for ½ to 1 minute (with agitation), the starch paste is heated in a water bath for 15 to 30 minutes. The mouth of the flask is kept covered by an inverted beaker during the heating period. Grinding exerts the favorable effect of keeping the starch in a well dispersed state for a long time, whereas without the grinding it soon forms a heavy sediment. This preparation keeps in an icebox for several weeks. However, it must be tested for reducing substances every week.

Acid sodium chloride solution. This contains 10 grams of NaCl and 3 cc. of 0.1 N HCl per liter. The presence of the acid is necessary for correction of the pH of blood serum or plasma which tends to rise above the upper limit of the optimum pH range (7.0 to 7.4) owing to loss of carbon dioxide during the preparatory manipulations.

Protein precipitants. A 5 per cent solution of $CuSO_4 \cdot 5H_2O$ and a 6 per cent solution of Na_2WO_4 are used for the deproteinization of plasma or serum.

Copper reagent of high alkalinity. Add in order 6 grams $CuSO_4 \cdot 5H_2O$., 25 grams Rochelle salt and 48 cc. N. NaOH. Dissolve and then add 35 grams sodium carbonate (anhydrous), 5 grams potassium iodide and 0.640 grams potassium iodate. Water to 1 liter.

Blood sample. Either oxalated plasma or serum may be used (not less than 7 cc.). Blood should be drawn at the height of the attack. Amylase activity, if high, is not significantly lost if kept in ice box over night. However, precipitation should be carried out as soon as possible.

PROCEDURE

1. Bring to 40°C. in a test tube, 5 cc. of starch paste and 2 cc. acid NaCl.
2. Add 1 cc. of plasma and incubate exactly 30 minutes at 40°C.
3. Immediately add 1 cc. of $CuSO_4$, mix, add 1 cc. sodium tungstate and mix immediately. (The copper sulfate does not fully stop diastase action.)
4. The plasma glucose is determined in separate analysis by mixing the following: 1 cc. plasma, 7 cc. water, 1 cc. 5 per cent copper sulfate, and 1 cc. 6 per cent sodium tungstate.
5. Filter both samples and determine the copper-reducing power by heating in a boiling water bath for exactly 20 minutes 5 cc. of filtrate and 5 cc. "high alkalinity" copper reagent.

6. The rest of the procedure follows the Shaffer-Hartmann technique for determining reducing substances in blood (J. B. C., 100, 695, 1933).

CALCULATION

Diastatic activity is reported as reducing power in terms of glucose.

Normal. 80 to 150 mgm. per cent. Values above normal which exceed 400 mgm. per cent require dilution of serum before incubation. In severe cases values up to 2 or 3000 may be found. The incubated sample contains original reducing substance plus preformed reducing substance. In terms of glucose: Glucose (incubated) mgm. per cent − blood glucose mgm. per cent = amylase mgm. per cent

A NEW METHOD OF MAKING SMEARS FOR HEMATOLOGIC, CYTOLOGIC AND BACTERIOLOGIC EXAMINATIONS

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The following method for making smears is intended chiefly for hematologic examinations, although it may also be utilized for certain cytologic and bacteriologic examinations.

Two slides are used. One drop of blood or of any other material to be examined is placed toward the edge of a slide (A) in the usual way. Then, as this slide is held at an angle of about 35°, the material is smeared on a second slide (B) from the center toward one end, until an evenly spread, *thin* film occupies half of the slide.

Three to five drops of the material to be examined are then placed on the first slide (A) in the usual way, and are smeared on the other half of the second slide (B) from the center to the end, until an evenly spread, *thick* smear occupies this half of the slide. One half of the slide (B) is thus covered with a thin film and the other half with a thick film of the material to be examined.

The advantages of this method are self-evident. The thin smear is useful for the demonstration of the morphology of erythrocytes. The thick portion is particularly useful in all varieties of leukopenia, in which it is impossible to make a differential white count from a thin smear, and in such conditions as malaria and other diseases in which parasites may be so infrequent that a thick smear is essential for their demonstration.

The author has found no note of a similar method elsewhere in the literature.

CORRECTION

Dr. Fabry L. Hawk, whose article *A Simplified Method for the Determination of Blood Urea* appeared in the September 1938 issue of the Technical Supplement, asks that the following correction be made: In the description for the preparation of Nessler's Solution, on page 155, the amount of pure iodine should read 55 grams instead of 110 grams.